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Characterization and Antimicrobial Synergy of Capsicum Oil Nanoemulsion with Colistin Against Gram-Negative Bacteria

Hesham A. Malak
Department of Biology, Faculty of Science, Umm Al-Qura University, Makkah, Saudi Arabia.
*E. mail: **hamalak@uqu.edu.sa**

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ABSTRACT

Colistin, a well-known Ab that combats gram-negative bacteria, has its antimicrobial effect restricted due to its nephrotoxicity side effect. Capsicum essential oil offers antimicrobial properties, but its poor solubility in water limits its usage in food. Incorporating it into a nanoemulsion can protect and enhance its application. The aim of our study is to examine the enhanced antimicrobial effect of capsicum oil nanoemulsion when combined with colistin against gram-negative bacteria. We used the agar well diffusion method to examine the antimicrobial effects of nanoemulsion, colistin, and their combination against *Escherichia coli* (ATCC 10536) and *Klebsiella pneumoniae* (ATCC 10031). TEM was used to examine the ultrastructure of the treated bacteria. Capsicum oil nanoemulsion droplets ranged in size from 41 to 123 nm, with a Z-potential of -25.8 mV and a PDI of 0.573, showing stability during our in vitro study. The combination of nanoemulsion with colistin exhibited a remarkable antimicrobial effect against *Klebsiella pneumoniae*, with an inhibition zone of 22±1.58 mm, compared to colistin alone, which had an inhibition zone of 18.60±1.14 mm. However, there was no difference in the inhibition zones for *E. coli* between the combined treatment and colistin alone, both showing an inhibition zone of 27.4±0.89 mm. TEM of gram-negative bacteria treated with both nanoemulsion and colistin showed clear cellular lysis and nuclear necrosis. Our results confirm that co-administration of colistin with capsicum oil nanoemulsion enhances the antimicrobial effect of colistin more effectively than its use alone.

INTRODUCTION

Bacterial diseases have long been a global health burden, and despite advancements in Ab development, resistance remains a significant challenge (Aslam *et al.*, 2021). Bacterial resistance can be attributed to modifications that prevent Ab from effectively targeting them. One strategy to overcome this challenge is to combine Ab with nanodrugs, which help them reach target sites more effectively and enhance their antimicrobial action (Yeh *et al.*, 2020).

Colistin is a well-known cationic Ab that binds to the negatively charged lipopolysaccharide lipid A present in the outer CW of gram-negative bacteria, causing damage that leads to cellular leakage and necrosis (Gai *et al.*, 2019). Unfortunately, long-term use of colistin can result in nephrotoxicity, which depends on the duration and dose of administration (Ordooei *et al.*, 2015; Lim *et al.*, 2010).

Furthermore, resistance among gram-negative bacteria is increasing markedly, limiting the prescription of colistin. However, recent research has established that combining colistin with nanodrugs enhances its antimicrobial power and reduces its toxic side effects (Liu *et al.,* 2018; Chen *et al.,* 2021; Mei *et al.,* 2013).

Traditional medicine in developing countries is a key target for disease control due to its availability and low cost (Morsy *et al.,* 2024). The unique chemical components of traditional medicine encourage scientists to examine it against drug-resistant bacteria (Akinpelu *et al.,* 2008). Capsicum annuum L. is a well-known traditional medicine used to treat stomachache, diarrhea, and dysentery symptoms (Tchiegang *et al.,* 2023). Extracts of Capsicum annuum L. have shown inhibitory effects against Pseudomonas aeruginosa, Staphylococcus aureus, Listeria monocytogenes, Bacillus cereus, and Salmonella typhimurium (Izah *et al.,* 2019). The fruits of C. frutescens var. minima are considered the source of aqueous and ethanolic extracts of Capsicum annuum L. through bending and drying (Izah, 2019). The disc diffusion method was used to examine the antimicrobial effect of Capsicum annuum L. methanolic extract against E. coli and S. aureus, while the agar well diffusion method tested the antimicrobial properties of the extract on S. aureus, P. aeruginosa, and E. coli (Gahongayire *et al.,* 2018). The antimicrobial effect of Capsicum annuum L. extract can be attributed to its rich content of carotenoids, vitamins C and E, alkaloids, phenolic components, and fatty acids such as linolenic, palmitic, and stearic acids (Bae *et al.,* 2012; Guo *et al.,* 2021; Srinivas *et al.,* 2023; Ciulu-Costinescu *et al.,* 2015).

The food industry tends to use nanoemulsions with a 100nm radius for the protection and encapsulation of lipophilic food contents, making them easier to deliver (McClements and Rao, 2011). Nanoemulsions improve physiological performance, oral bioavailability, water solubility, and thermal stability of food (Huang *et al.,* 2010). Our study is designed to examine the in vitro antimicrobial effect of co-administering capsicum oil nanoemulsion with colistin Ab against gram-negative bacteria, enhancing its antimicrobial effect and minimizing its side effects.

MATERIALS AND METHODS

Preparation of Capsicum oil Nanoemulsion:

The capsicum oil in water (capsicum oil from Pure Company, Giza, Egypt) was formulated according to the procedure described by (Ibrahim *et al.,* 2021). For the preparation of capsicum oil nanoemulsion, a mixture of one milliliter of capsicum oil and Tween 80 surfactant was slowly added to ten milliliters of distilled water at twenty-five Celsius using a magnetic stirrer, with the water being added at a rate of 1.0 mL per minute. The emulsion was then dispersed using an ultrasonic bath (Sonix, Springfield, VA, USA, SS101H230) for half an hour. Additionally, the emulsion was homogenized using an ultrasonic probe (Serial No. 2013020605, Model CV 334) attached to the homogenizer (Sonics Vibra-cell™, Model VC 505, Inc., USA), set at sixty percent amplitude, with a five-minute timer, and a one second ON/one second OFF cycle for nanoemulsion formation.

Characterization of Capsicum oil Nanoemulsion:

A Transmission Electron Microscope (TEM, JEOL JEM-2100, JEOL LTD, Tokyo, Japan) was used to visualize the internal morphology of the capsicum oil nanoemulsion at one-hundred and sixty kilovolts. Soft imaging and digital micrograph viewer software were used to analyze the captured images. The nanoemulsion's PDI, surface charge (Z-potential), and vesicular size average (Z-average) were assessed using a Zetasizer

analyzer (Malvern Instruments, Malvern, United Kingdom).

Microbial susceptibility testing: Agar well diffusion method:

The antimicrobial activity of colistin and capsicum oil nanoemulsion was evaluated using the method of agar well diffusion, which is similar to the disk diffusion test. The surface of the agar plate was inoculated by dispersing the tested bacteria over the surface. A 9 mm diameter hole was then perforated using a sterile cork borer, and 50µL of colistin or capsicum oil nanoemulsion was added to the well. The agar plates were incubated for one day, allowing the nanoemulsion and colistin to diffuse through the agar medium and inhibit the growth of the tested bacteria. The tested bacteria included gram-negative bacteria such as Escherichia coli (ATCC 10536) and Klebsiella pneumoniae (ATCC 10031) (Murray *et al.,* 1998; Davidova *et al.,* 2024).

Transmission Electron Microscopy Examination of Bacterial Samples:

The ultrastructural changes in Klebsiella pneumoniae and E. coli samples, due to treatment with colistin and capsicum oil nanoemulsion, were evaluated using a Transmission Electron Microscope (TEM). For the preparation of ultrathin sections used in TEM examination, bacterial fixation followed the method established by Morris, (1965). The bacterial cells of Klebsiella pneumoniae and E. coli were preserved at four-degree Celisus overnight, then washed three times for fifteen minutes with 0.1 M sucrose and 0.1 M sodium phosphate buffer. They were then postfixed with 2% sodium phosphate buffer osmium tetroxide for ninety minutes, followed by washing with 0.1 M sodium phosphate buffer for fifteen minutes three times. The cells were dehydrated with 50% ethanol for fifteen minutes twice. Subsequently, the cells were contrasted with 0.5% uranyl acetate, 1% phosphotungstic acid, and 70% acetone overnight at 4°C. The cells were then dehydrated using an ascending scale of ethanol (80%, 90%, 96%, 100%) twice for fifteen minutes each. After that, the cells were treated with 2:1, 1:1, and 2:1 mixtures of acetone and Epon for 30 minutes each. The cells were then placed in pure Epon solution at 4°C overnight, followed by a fresh Epon solution. Polymerization was done by incubating the cells at 65°C for two days. The samples were then cut into 50 to 100 nm sections using an ultramicrotome. The sections were placed on copper grids and post-contrasted with 8% uranyl acetate for ten minutes, followed by 0.7% lead acetate with 0.9% sodium citrate for five minutes, and dried for fifteen minutes. Finally, the sections were examined using a Transmission Electron Microscope (El-Gazzar and Ismail, 2020; El-Gazzar *et al.,* 2020; El-Bahr *et al.,* 2021).

Statistical Analysis:

All study experiments were performed in triplicate, and the data were evaluated using one-way ANOVA to estimate M±SD. $p < 0.05$ was considered.

RESULTS AND DISCUSSION

1-Capsicum Oil Nanoemulsion Characterization:

Transmission electron microscopic examination of capsicum oil nanoemulsion showed near-spherical particles with no aggregation, and an average size of 95.25 nm (Fig. 1A, B). The polydispersity index (PDI) and ZP values of the capsicum oil nanoemulsion were 0.573 and -25.86 mV, respectively (Fig. 1C, D). The absorption of most oils from the gastrointestinal tract can be enhanced by transforming them into nanoemulsion form, overcoming their lipophilic characteristics. In this study, we used an oil-in-water nanoform to prepare the capsicum oil nanoemulsion with a size of 95 nm, a negative ZP of -25.86 mV, and a PDI of 0.573. This confirms that nanoemulsions with small-sized particles, surface negative charges, and hydrophilic characteristics can cross the intestinal mucosal layer (Guo *et al.,* 2021). It has been reported that droplets less than 200 nm are considered nanoemulsions (McClements, 2012; Tadros *et al.,* 2023; McClements and Jafari,

2018). Nanoemulsions with smaller particles have shown high stability, which affects their antimicrobial, physicochemical, and sensory properties, as well as their overall stability (Haghju *et al.*, 2016). Previous research on the fabrication of oleoresin capsicum with chitosan/alginate polymers improved its bioavailability (Choi *et al.*, 2013). Our study's results align with previous work by (Choi *et al.*, 2013), which documented that capsaicin-loaded nanoemulsions had a particle size of 106.7 ± 0.36 nm, a ZP of -25.4 ± 0.36 mV, and a PDI of 0.71 ± 0.03.

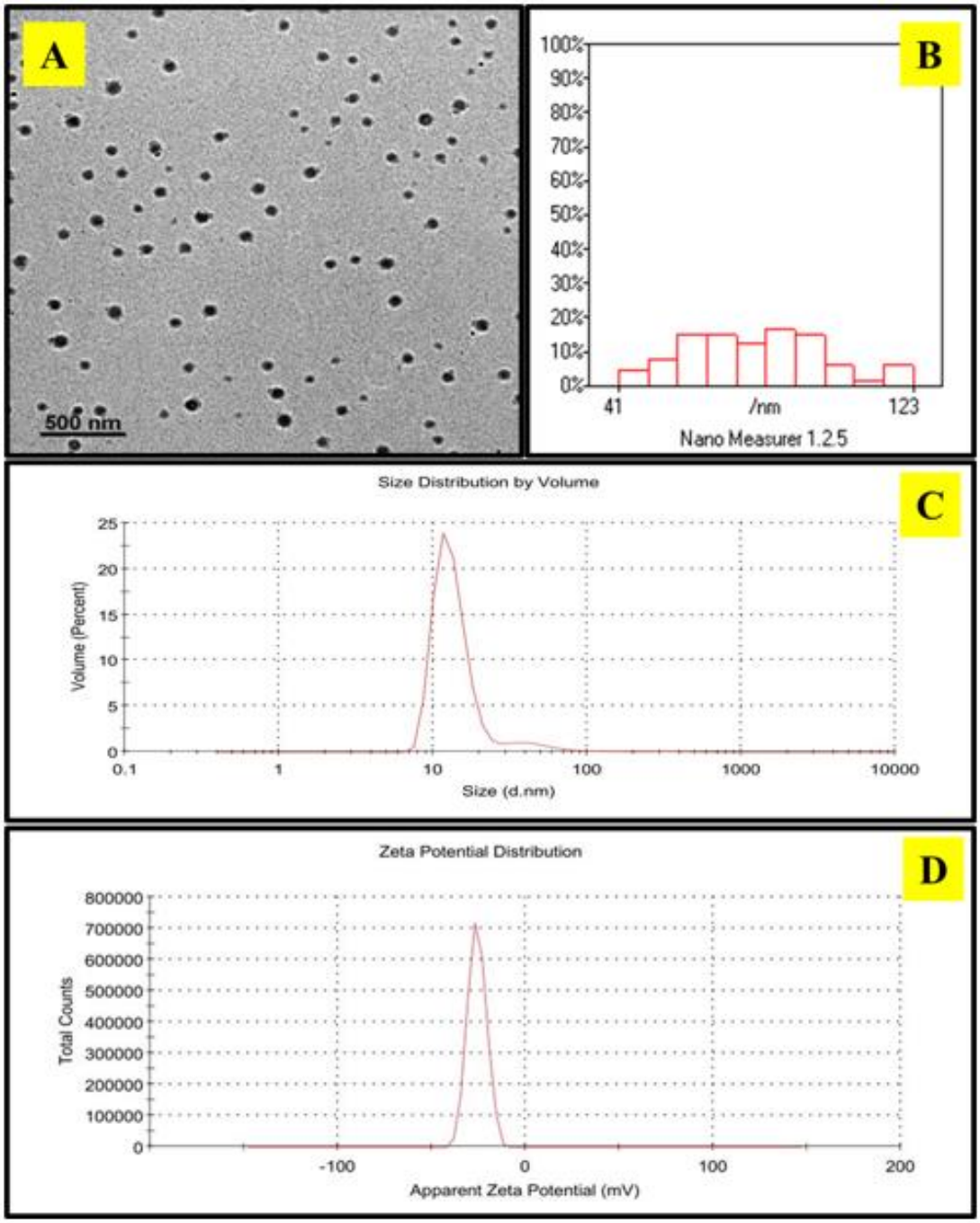

Fig. 1. (A)The morphology of capsicum oil Nano emulsion by TEM showing nearly spherical particles, (B) histogram shows that most particles cluster around 41 to 123 nm, with a good distribution, C) Zeta size distribution by volume. D) Z-potential distribution. The values of Z-average size, PDI, and ZP of capsicum oil Nano emulsion were found to be 95.25 nm, 0.573, and -25.8.6mV respectively.

2. Antimicrobial Effect of Combined Capsicum Oil Nanoemulsion with Colistin:

In this study, the agar well diffusion test revealed that capsicum oil nanoemulsion produces no inhibition zone in either *E. coli* or *Klebsiella pneumoniae*. However, a combination of capsicum oil nanoemulsion with colistin produces a larger inhibition zone of 22±1.58 mm in *Klebsiella pneumoniae* compared to the colistin-only zone of 18.60±1.14 mm. There was no difference in the inhibition zone diameter between the combined treatment and colistin alone in the *E. coli* well agar (Table 1, Fig. 2). Capsicum oil nanoemulsion itself did not offer an antimicrobial effect against *Escherichia coli* (ATCC 10536), which is consistent with a previous study by Lima et al. (2024). They reported that a nanoemulsion of sodium alginate with pink pepper essential oil did not show antibacterial effects against gram--ve pathogen *E. coli* and *Salmonella sp*. Conversely, the nanoemulsion exhibited antimicrobial effects against gram-+ve pathogens *S. aureus* and *L. monocytogenes*. In contrast, a study by El-Naggar *et al.* (2020) stated that capsicum nanoemulsion with a high concentration of capsicum exhibited antimicrobial activity against gram-negative bacteria *E. coli*. This discrepancy can be explained by the dose-dependent nature of the antimicrobial effect of nanoemulsions. Moreover, the double-layer lipopolysaccharide of gram-negative bacteria acts as a barrier against antimicrobial agents (Nazzaro *et al.,* 2013; Donsì and Ferrari, 2016). However, a study by Salvia-Trujillo et al. (2015) reported that sodium alginate nanoemulsion with clove oil and lemongrass exhibited inhibition against *E. coli*. This indicates that the antimicrobial activity of essential oils is affected by the concentration and the active compounds present (Moore-Neibel *et al.*, 2013). Interestingly, the combination of capsicum oil nanoemulsion with colistin showed a larger inhibition zone in *Klebsiella pneumoniae* (ATCC 10031) compared to the colistin-only zone. This is in accordance with a previous study by Muenraya *et al.* (2022), which reported that silver nanoparticles conjugated with colistin exhibited a strong antimicrobial effect against gram-negative bacteria *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and E. *coli*, producing a larger inhibition zone in the agar well diffusion method than the colistin-treated zone. The antimicrobial activity of the nanoemulsion-colistin combination can be explained by the negative charge on the surface of the nanoemulsion, which prevents it from binding to the outer bacterial membrane. However, colistin, with its positive cationic charge, has a strong affinity for the lipopolysaccharide of gram-negative pathogens, facilitating the binding and penetration of the capsicum oil nanoemulsion.

Table 1. Antibacterial activity of capsicum oil nanoemulsion with colistin against pathogenic bacteria.

Microorganisms	Inhibition zones in mm		
	Capsicum oil nanoemulsion (11.2µg/mL)	**Capsicum oil nanoemulsion (11.2 µg/mL) + Colistin (55.7µg/mL)**	**Colistin (55.7µg/mL)**
Escherichia coli (ATCC 10536)	-ve	27.4±0.89 mm	27.4±0.89mm
Klebsiella pneumonia (ATCC 10031)	-ve	22±1.58 mm	18.60±1.14mm

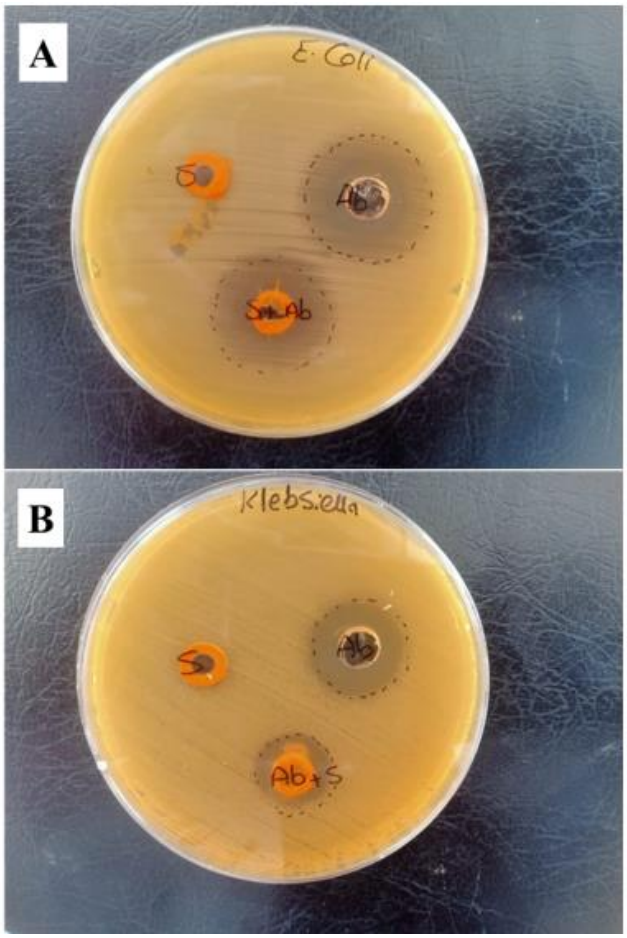

Fig.2. Agar well diffusion for analyze for capsicum oil nanoemulsion and colistin and them combination. The examined gram-negative bacteria (A) Escherichia coli, and (B) Klebsiella pneumonia. The letter S represented capsicum oil nanoemulsion, Ab represents colistin Ab, Ab+s represents combined treatment.

Transmition Electron Microscopic Examination of Examined Bacteria For Different Treatments:

TEM examination of ultrathin sections of examined bacteria treated with nanoemulsion revealed the normal structure of the bacteria, with intact PM, PS, and CW, along with homogeneous nucleoids (Fig. 3A, B). In contrast, colistin-treated *E. coli* showed disarrangement in shape with some cells exhibiting shrinkage. *Klebsiella pneumoniae* treated with colistin displayed mesosome-like structures and the formation of non-membranous enclosed bodies. Interestingly, the combined treatment with capsicum nanoemulsion and colistin exhibited a strong antimicrobial effect obvious on bacterial ultrastructure. This was evident as completely lysed bacteria and necrosis in *E. coli*, and necrotic cells with intracellular nuclear lysis in *Klebsiella pneumoniae*. This confirms the enhanced antibacterial effect of the combined application compared to treatment with colistin or nanoemulsion alone. The necrosis of bacterial cells in the combined treatment of nanoemulsion and colistin can be explained by the interaction of the positive cationic peptide of colistin with the bacterial membrane's lipopolysaccharide. This interaction facilitates bacterial penetration by the combined nanoemulsion-colistin treatment, causing lysis of bacterial cells. This finding is supported by previous research, which have shown that the strong antibacterial effect of combined colistin and nanoparticles is related to bacterial membrane degeneration (Gai *et al.*, 2019; Lee and Jun, 2019).

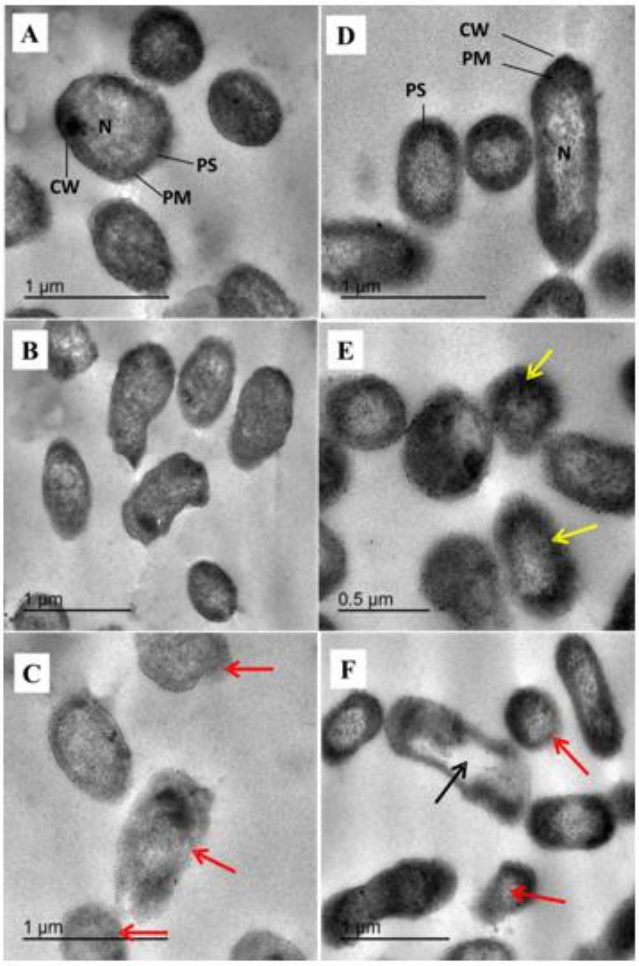

Fig. 3. TEM micrographs of Bacterial cells where: (A) E. Coli and (D) Klebsiella pneumonia capsicum nanoemulsion bacterial groups showing rounded cells with normal appearance of them compartments, well-defined CW, PM, and periplasmic membrane (PS). Intracellular DNA showed a homogenous electron density like nucleoid (N). (B) E. coli Colistin treated group showing Bacterial cells loss its normal shape and some cells were shrinked. (C) E. coli Colistin + capsicum oil nanoemulsion group showing completely lysed cells reached to a necrosis (Red arrow). (E) Klebsiella pneumonia (Colistin treated group) showed mesosomes like structures and non-membranous enclosed bodies (yellow arrow). (F) Klebsiella pneumonia Colistin+ Capsicum oil nanoemulsion treated group showing intracellular nuclear content lyses (black arrow), the CW with different thicknesses and invagination of PS. Also appear of necrotic cells (Red arrow). Our study faces several limitations. Firstly, the findings are based on in vitro experiments and may not fully translate to in vivo conditions due to the complexity of biological systems. Further studies using animal models and clinical trials are necessary to validate the efficacy and safety of the combined treatment in living organisms. Secondly, while our study suggests that the combination may reduce the nephrotoxic effects of colistin, comprehensive studies are needed to confirm this benefit. Understanding how nanoemulsion modulates colistin's toxicity is essential for its clinical application.

CONCLUSIONS

In our work, capsicum oil in nanoemulsion (water-oil interface) demonstrated stability throughout the study due to its small particle size and negative surface charge. The combination of colistin and capsicum nanoemulsion revealed a stronger antimicrobial effect against gram-negative bacteria compared to treatment with nanoemulsion or colistin alone. This

effect is attributed to the interaction between colistin and nanoemulsion with the bacterial membrane, facilitating its disruption and penetration, leading to damage to bacterial nuclei and cellular lysis. The enhanced antimicrobial effect of capsicum nanoemulsion in combination with colistin against gram-negative bacteria opens the door for further examination of its impact on the nephrotoxicity side effect of colistin, especially since we confirmed its positive antimicrobial efficacy.

Abbreviations list:
ATCC: American Type Culture Collection
ANOVA: Analysis of Variance
PDI: Polydispersity Index
TEM: Transmission Electron Microscopy
Z-average: Zeta-average diameter
Z-potential: Zeta potential
Ab: Antibiotic
ATCC: American Type Culture Collection
CW: Cell Wall
DNA: Deoxyribonucleic Acid
mV: Millivolt
nm: Nanometer
PM: Plasma Membrane
PS: Periplasmic Space
SD: Standard Deviation

DECLARATIONS:

Ethical Approval: Not applicable.

Conflicts of Interest: The author declares no conflicts of interest.

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Availability of Data and Materials: All datasets analyzed and described during the present study are available from the corresponding author upon reasonable request.

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